

**PCT**WORLD INTELLECTUAL PROPERTY ORGANIZATION  
International Bureau

## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification <sup>6</sup> :</b> <b>G01N</b>	<b>A2</b>	<b>(11) International Publication Number:</b> <b>WO 97/03345</b> <b>(43) International Publication Date:</b> 30 January 1997 (30.01.97)
<b>(21) International Application Number:</b> PCT/GB96/01660 <b>(22) International Filing Date:</b> 12 July 1996 (12.07.96)  <b>(30) Priority Data:</b> 9514325.1 13 July 1995 (13.07.95) GB  <b>(71) Applicant (for all designated States except US):</b> ROYAL FREE HOSPITAL SCHOOL OF MEDICINE [GB/GB]; Rowland Hill Street, London NW3 2PF (GB).  <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only):</b> EMERY, Vincent, Clive [GB/GB]; Department of Virology, Division of Pathology & Communicable Diseases, Rowland Hill Street, London NW3 2PF (GB). GRIFFITHS, Paul [GB/GB]; Department of Virology, Division of Pathology & Communicable Diseases, Rowland Hill Street, London NW3 2PF (GB).  <b>(74) Agents:</b> BRASNETT, Adrian, Hugh et al.; J.A. Kemp & Co., 14 South Square, Gray's Inn, London WC1R 5LX (GB).		<b>(81) Designated States:</b> JP, US, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).  <b>Published</b> <i>Without international search report and to be republished upon receipt of that report.</i>
<b>(54) Title:</b> DETECTION AND QUANTIFICATION OF HUMAN HERPESVIRUS 7 BY ENZYMIC AMPLIFICATION  <b>(57) Abstract</b>  An isolated nucleic acid molecule comprising: (i) a primer portion consisting of a contiguous sequence of from 10 to 50 nucleotides capable of hybridising to (a) the target nucleic acid molecule represented by SEQ. ID No. 1, or (b) to the complementary stand thereof; and optionally (ii) a further portion comprising from 1 to 25 nucleotides joined to and immediately 5' to the 5' end of the primer portion.		

**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AM	Armenia	GB	United Kingdom	MW	Malawi
AT	Austria	GE	Georgia	MX	Mexico
AU	Australia	GN	Guinea	NE	Niger
BB	Barbados	GR	Greece	NL	Netherlands
BE	Belgium	HU	Hungary	NO	Norway
BF	Burkina Faso	IE	Ireland	NZ	New Zealand
BG	Bulgaria	IT	Italy	PL	Poland
BJ	Benin	JP	Japan	PT	Portugal
BR	Brazil	KE	Kenya	RO	Romania
BY	Belarus	KG	Kyrgyzstan	RU	Russian Federation
CA	Canada	KP	Democratic People's Republic of Korea	SD	Sudan
CF	Central African Republic	KR	Republic of Korea	SE	Sweden
CG	Congo	KZ	Kazakhstan	SG	Singapore
CH	Switzerland	LI	Liechtenstein	SI	Slovenia
CI	Côte d'Ivoire	LK	Sri Lanka	SK	Slovakia
CM	Cameroon	LR	Liberia	SN	Senegal
CN	China	LT	Lithuania	SZ	Swaziland
CS	Czechoslovakia	LU	Luxembourg	TD	Chad
CZ	Czech Republic	LV	Latvia	TG	Togo
DE	Germany	MC	Monaco	TJ	Tajikistan
DK	Denmark	MD	Republic of Moldova	TT	Trinidad and Tobago
EE	Estonia	MG	Madagascar	UA	Ukraine
ES	Spain	ML	Mali	UG	Uganda
FI	Finland	MN	Mongolia	US	United States of America
FR	France	MR	Mauritania	UZ	Uzbekistan
GA	Gabon			VN	Viet Nam

## DETECTION AND QUANTIFICATION OF HUMAN HERPESVIRUS 7 BY ENZYMIC AMPLIFICATION

This invention was made with US government support under grant number  
5 A133389-02 ("significance of HHV6-7 for immunocompromised patients") awarded  
by the National Institutes of Health, Bethesda, Maryland 20892, USA. The US  
Government has certain rights in the invention.

This invention relates to a method for detecting and quantifying the presence of  
10 human herpesvirus 7 (HHV-7) in a sample, and to nucleic acid sequences useful in  
such a method.

HHV-7 is a member of the family Herpesviridae and is related to but genetically  
distinct from human herpesviruses 6A and 6B (HHV-6A and HHV-6B). HHV-7  
15 was first isolated in 1989 and subsequently found to be present in the saliva of  
from 75% to over 90% of healthy adult humans (Frenkel *et al* (1990): PNAS 87,  
748-752; Wyatt *et al* (1991): J. Virol. 65(11), 6250-6265).

HHV-7 has been found to cause an *exanthem subitum*-type illness in some children,  
20 one symptom of this illness being the development of rashes and acute febrile  
illness. At present, other disease symptoms attributable to HHV-7 have not been  
identified but, by analogy with other human herpesviruses, HHV-7 can be  
expected to cause disease, especially in immunocompromised hosts, such as organ  
transplant recipients. Therefore, it is desirable to be able to detect and quantify  
25 the presence of HHV-7 in samples of human tissue and bodily fluids. Until now,  
methods of detecting HHV-7 have been serological in nature, relying on the use of  
antibodies to HHV-7 proteins. These methods are, however, unsatisfactory. This  
is because they fail to quantify HHV-7, or have low specificity for HHV-7 with  
respect to other viruses such as HHV-6A/B.

30

The inventors have identified a sequence of HHV-7 DNA that is unique to this  
virus and not found in other related viral genomes although it codes for a protein

that has some sequence homology with the product of KA3L gene of HHV6. The unique sequence is a 193 base pair fragment of HHV-7 DNA, and was identified by digestion of purified HHV-7 DNA with a restriction endonuclease, followed by cloning the restriction fragments into a plasmid vector. Using the sequence of this  
5 193 base pair "target" fragment, it has been possible to synthesise a variety of primers that hybridise to it over part of its length. These can be used in Polymerase Chain Reaction (PCR), including nested PCR reactions, to amplify the 193 base pair fragment.

10 The inventors have also prepared a mutant DNA control sequence based on the 193 base pair fragment. This construct has internal substitutions that provide an additional restriction site (for the restriction endonuclease SmaI) that is absent from the target sequence. Primers suitable for construction of the mutant sequence by PCR amplification have also been synthesised.

15 The mutant control sequence can therefore act as a target sequence mimetic for the 193 base pair fragment in any PCR reaction performed on a sample containing the 193 base pair sequence and the control sequence. This forms the basis of a method of detecting and quantifying the presence of the 193 base pair fragment in samples,  
20 for example samples of human tissue or bodily fluids, and thus quantifying HHV-7 in these samples. A known amount of the control sequence is added to the sample; a pair of primers is also added and PCR is performed. As the target and control sequences are very similar, and preferably identical at the primer binding sites, one pair of primers allows the amplification of both the 193 base pair target  
25 sequence and the control sequence by PCR. Cleavage of the mutant sequence with the restriction endonuclease SmaI, which does not cleave the 193 base pair wild-type sequence, followed by quantification of the cleavage products then allows the amount of the target sequence present to be determined as the amount of the control sequence added is known. This, in turn, allows the presence of the  
30 HHV-7 virus in the sample be quantified as each HHV-7 genome comprises one target sequence.

As the target sequence is unique to HHV-7, this method can be used to quantify HHV-7 accurately in samples, thus potentially providing information as to the likelihood of the sample donor suffering from the symptoms caused by the virus.

5 Accordingly, the invention provides:

- An isolated nucleic acid molecule comprising (i) a primer portion consisting of a contiguous sequence of from 10 to 50 nucleotides capable of hybridising to (a) the target nucleic acid molecule represented by SEQ. ID No. 1 or (b) to the
- 10 complementary stand thereof; and optionally (ii) a further portion comprising from 1 to 25 nucleotides joined to and immediately 5' to the 5' end of the primer portion;

- a pair of nucleic acid molecules as defined above wherein one nucleic acid molecule
- 15 has a primer region of type (a) as defined above and the other nucleic acid molecule has a primer region of type (b) as defined above; and wherein the two primers in combination are capable of amplifying the target nucleic acid molecule represented by SEQ. ID No. 1 or a section thereof in a polymerase chain reaction;

- 20 a pair of nucleic acid molecules as defined above that hybridise respectively to the target nucleic acid sequence of SEQ. ID No. 1 and its complementary stand in such positions that the 5' end of the each primer region hybridises at a location that is from 0 to 50 nucleotides 3' to the 5' end of the sequence of SEQ. ID No. 1 or the complementary strand thereto;

25

A method of determining the amount of a target nucleic acid having the sequence shown in SEQ. ID No. 1 in a sample which method comprises:

- (i) mixing the sample with a predetermined amount of control nucleic acid;
- 30 (ii) bringing the mixture formed in (i) into contact with at least one nucleic acid primer as defined above that is capable of hybridising to the target nucleic acid and at least one primer as defined above that is capable

of hybridising to the control nucleic acid;

(iii) performing a nucleic acid amplification reaction, which reaction requires the presence of the primers defined in (ii) to amplify the target nucleic acid sequence or a section thereof and the control nucleic acid or a section thereof;

(iv) determining the relative quantities of the amplified control and target nucleic acids; and

(v) calculating from the determination of (iv) the amount of target nucleic acid in the sample;

An isolated nucleic acid sequence selected from the sequences shown in SEQ ID No. 1 and SEQ ID No. 2; or the complementary strands thereof; and

A kit for quantification of human herpesvirus 7 (HHV-7) in a sample by means of a method as defined above which kit comprises:

(i) a control nucleic acid as defined above;

(ii) one or more pairs of primers as defined above that are suitable for amplifying both the target and control nucleic acid sequences.

## BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Construction of the 183bp control sequence of SEQ. ID. No: 2.

Figure 2. Results of qualitative PCR on saliva samples, using the inner and outer primers of the invention.

Figure 3. Demonstration that the assay of the invention gives accurate data on samples with known genomic equivalents.

Figure 4. Prevalence of HHV-7 DNA in blood samples from HHV-7 seropositive individuals varies according to the quantity of input DNA used for analysis.

Figure 5 & 6. Longitudinal analysis of HHV-7 viral load in saliva and peripheral blood of healthy volunteers shows that the amount of virus remains relatively constant over time.

- 5 Figure 7, 8 & 9. Qualitative detection of HHV-7 in live, renal and bone marrow transplant recipients.

Figure 10. HHV-6 and HHV-7 blood viral load versus clinical syndrome.

- 10 Figure 11. HHV-7 load in organs at death.

The target sequence that is amplified in methods of the invention is the 193 base pair sequence of SEQ. ID. No. 1 or a section of it, preferably the entire 193 base pair sequence or the section thereof shown in SEQ. ID. No. 9. Where the target  
15 sequence is a section of the sequence of SEQ. ID No. 1, the section may be of any length provided that the section in question is unique to the HHV-7 genome, in the sense that it is not found in the genomes of other viruses, particularly HHV-6A and HHV-6B, that infect human cells, or in the human genome. For example, a section may comprise up to 50, up to 100, up to 150 or up to 190 of  
20 the nucleotides of SEQ. ID No. 1.

The control sequence that is amplified in methods of the invention may be any suitable sequence that allows quantification of the target sequence. The control nucleic acid may be any suitable nucleic acid, eg. a single or double stranded  
25 fragment or a sequence contained within a vector, for example a plasmid vector. Preferably, the control nucleic acid is chosen so that it will undergo amplification at a substantially identical rate to that of the nucleic acid in the sample which is to be analyzed. Following amplification, measurement of the amount of amplified control sequence relative to the amount of amplified target sequence from the  
30 sample allows the quantity of nucleic acid originally present in the sample to be determined.

Accordingly, it is preferred that the control sequence is of similar length and topology to the target sequence. Thus, the control sequence may be of any length. For example, lengths of up to 50, up to 100, up to 250, up to 200 or up to 300 base pairs are preferred with lengths in the region of from 100 to 250bp being  
5 more preferred and lengths of 180 to 220bp being particularly preferred. The most preferred control sequence, as shown in SEQ ID No. 2, has a length of 183 base pairs, as it was obtained by introducing a SmaI recognition site into the sequence of SEQ. ID. No. 1.

10 Also, it is preferred that a control sequence of the invention has a high degree of sequence homology to the target sequence. Preferably, the control sequence has at least 70%, at least 80%, at least 90%, at least 95% or at least 99% homology with the target sequence.

15 Further, it is preferred that a control sequence of the invention has a similar topology to the target sequence. Thus, linear control sequences are preferred.

The difference between the target and control sequences may be in the nature of one or more nucleic acid deletions, substitutions or insertions, or may arise from  
20 any other type of nucleic acid rearrangement or sequence alteration.

In a preferred embodiment of the invention, the control nucleic acid differs from the target nucleic acid by the presence of at least one predetermined restriction enzyme recognition sequence (restriction site). Such a restriction site can be  
25 generated, for example, using a copy of the original target sequence which is altered by site-directed mutagenesis or by using oligonucleotide primers complementary to the target sequence at all residues except those which need changing to construct the desired restriction site. Such primers may have any appropriate sequence and the primer pair represented by SEQ. ID. Nos. 7 and 8  
30 are examples of suitable sequences. These primers can be used to introduce a SmaI site into the sequence of SEQ. ID. No. 1, and other primers could be used to introduce other restriction sites. Following PCR amplification of the target and



control sequences, the reaction mixture (or a portion thereof) is brought into contact with a restriction enzyme capable of cutting the predetermined restriction site or sites. In this way, the uncut amplified target sequence can be resolved, eg. by electrophoresis, from the two or more fragments of the cut amplified control sequence; ultimately allowing quantification of the target sequence.

It is preferred that the predetermined restriction site in the control nucleic acid is a restriction site that is unique to the control sequence, i.e. only two fragments of DNA will be produced upon digestion of the control fragment. Desirably, the predetermined restriction site is centrally located in order that the two fragments produced by digestion are of the same or of a similar size, eg. within 15, preferably 5, nucleotides in length of each other. This will mean that the two fragments produced will appear as a single band on a gel following electrophoresis. Quantitative analysis of the band will provide a determination of the total amount of amplified control DNA.

Other configurations of restriction sites are also possible. For example, the control nucleic acid may contain, for example, 2, 3, 4 or more restriction sites, one or more of which can be cut following PCR. Such multiple restriction sites may be recognition sites for the same enzyme or for two or more different enzymes. They may be located in a manner such that digestion with the appropriate enzymes produces fragments of a substantially similar size or of different sizes. Such configurations could be used to provide an internal control for the efficiency of the restriction digest or to provide fragments of convenient size for resolution. For example, a restriction site unique to the selected region of the target nucleic acid which, when cut, will divide the selected region into two unequal fragments could be eliminated from part of the control sequence region and the same restriction site introduced at a central location in the said control sequence. Following PCR and digestion with an enzyme capable of cutting this centrally located site in the control region followed by resolution by electrophoresis, three different size fragments will be seen. The amount of DNA in all three fragments may be measured and the amount of target DNA quantified. If for any reason the

restriction digest is incomplete, a further amplified fragment will be seen.

However, this will not affect the quantification of target nucleic acid, which can be determined simply by the ratio of digested fragments.

- 5 A further method of controlling the digest is to add to the sample, prior to digesting, a further DNA fragment containing a restriction site for the restriction enzyme which is to be used for digesting of the target DNA which is of a size such that it will not, either as a complete fragment or following digestion, be superimposed over digested or undigested control or target nucleic acid in the  
10 resolution step of the method of the invention. This can be used to monitor the efficiency of the digest. The ratio of undigested to digested further DNA can be used to correct for any incomplete restriction digests.

- Although the embodiment of the invention discussed above envisages that the  
15 control region will be altered to introduce a predetermined restriction site, the invention also includes altering the control DNA to eliminate a restriction site, should a convenient restriction site occur in the target nucleic acid. Where a suitable restriction site occurs in the natural target nucleic acid, primers substantially equidistant from this site can be selected in order that the  
20 predetermined restriction site is centrally located in the selected region.

- In control sequences of the invention, any type of restriction site may be exploited in the manner outlined above. In one preferred control sequence, as shown in SEQ. ID. No. 2, a SmaI site has been introduced. The primer portions of these  
25 nucleic acids are capable of hybridizing to sections of both the target and control sequences, owing to the high sequence homology between the target and control sequences. In principle, such hybridization may occur with any section of the target sequence as long as the primer portion is capable of hybridizing to both the target and control sequences and initiating amplification of them. Preferably, the  
30 section over which hybridization occurs comprises at least 10, more preferably at least 15 to 20, for example 16, 17, 18 or 19; at least 30; or at least 50 to 100 nucleotides. More preferably, the primer portions of the nucleic acids of the

invention comprise from 10 to 15 nucleotides. The sections of the target sequence with which hybridization occurs may be located in any part of the target sequence; i.e. they may be within the sequence or at one end of it.

- 5 Optionally, the nucleic acids of the invention may also comprise a further portion, especially if the section to which the primer portion hybridizes is at the 5' end of the target sequence. The optional further portion may therefore comprise nucleotides that extend beyond the end of the target or control sequence. The length of the further portion may be up to 25, for example, up to 20, up to 18, up to 10 or up to 5 nucleotides. The sequence of the further portion may be of any nature. For example, it may be native HHV-7 nucleic acid sequence or a sequence with substantial sequence homology thereto. Alternatively, the sequence of the further portion may be an unrelated (non-HHV-7) sequence. For example, the further portion may be an amplifier strand as discussed in EP-A-0-317,077. This will allow detection of HHV-7 DNA by addition of a multimer probe comprising a first nucleic acid sequence complementary to the amplifier strand and two or more (eg. 4, 5, 6 or 7) sequences complementary to a separate labelled nucleic acid probe.
- 20 It is preferred that the primer portions will be perfectly complementary to the section of the target sequence to which they hybridise, in the sense that every nucleotide will base pair with the one with which it pairs most stably (A with T or U; C with G). However, small deviations from this rule may be allowed, so long as they do not prevent the primer from hybridising with the target and control sequences and initiating amplification. Thus, the primer portion may be, for example, up to 70%, up to 80%, up to 90%, up to 95% or up to 99% complementary to the relevant section of the target or control sequence.

- Preferred primers capable of hybridising to both the target (SEQ. ID. No. 1) and control sequences (e.g. SEQ. ID No. 2) of the invention are the primers of SEQ. ID. Nos. 3, 4, 5 and 6.

Preferably, the primers of the invention will be in isolated form, for example in aqueous solution. Thus, in samples of the primers, at least 90% at least 95% or at least 99% of the nucleic acid in the sample will typically be a single nucleic acid of the invention, or a pair of primers capable of amplifying of the target and control sequences in a PCR.

In a preferred embodiment of the invention, pairs of nucleic acids as described above are employed. Any two nucleic acids of the invention may form such a pair as long as the primer portion of one of them is capable of hybridizing to the target nucleic acid of SEQ. ID. No. 1 and the primer portion of the other is capable of hybridizing to the complementary strand to the target sequence, and as long as the two nucleic acids in combination are capable of initiating amplification of the target and control sequences or sections thereof. Thus, in preferred primer pairs of the invention, the two primer portions hybridize with the target nucleic acid and its complementary strand towards the 5' end of each strand. Accordingly, it is preferred that the nucleic acid molecules hybridize at a location that is from 0 to 50 nucleotides 3' to the 5' end of the sequence of SEQ. ID. No. 1 of the complementary strand thereto. For example, it is preferred that the 5' end of each primer portion hybridizes at a location that is from 0 to 10, from 10 to 20, from 20 to 30, from 30 to 40 or from 40 to 50 nucleotides 3' to the 5' end of the sequence of SEQ. ID. No. 1 or of the complementary strand thereto.

One preferred primer pair of the invention is provided by the combination of the nucleic acid molecules of SEQ. ID. No. 3 and SEQ. ID. No. 4. Another preferred pair is represented by SEQ. ID. No. 5 and SEQ. ID. No. 6.

The invention further provides methods for detecting and quantifying the occurrence of HHV-7 in samples. Typically, these samples will comprise human tissue, cells or bodily fluids or extracts derived from such tissues, cells or fluids. Thus, for example, methods of the invention may be used to detect and/or quantitate the number of viral genomes present in a sample, quantitative methods being preferred.

Thus, methods of the invention can detect and or quantify HHV-7 under a variety of circumstances. For example, it is desirable to detect and/or quantify HHV-7 in samples taken from patients who may be infected with HHV-7. Also, methods of the invention can be used to evaluate the effectiveness of antiviral drugs. In this case, cell cultures comprising HHV-7 can be assayed using methods of the invention, the resulting information being used to determine what concentration of antiviral agent to use.

Methods according to the invention rely on amplifying target and control nucleic acids as described above by any suitable method, PCR being a preferred method. In such methods, one or more primers as described above are used to initiate amplification. Typically, primers are employed in pairs for the amplification of double stranded nucleic acid with one primer binding to a site towards the 5' end of each strand.

In general, primers, preferably primer pairs as described above, are added to the sample, as well as the control sequence. As the target and control sequences have high sequence homology, and are preferably identical at the sites to which the primers bind, one pair of the primers hybridises to both target and control sequences. Thus, PCR leads to amplification of both sequences.

For the analysis and quantification of the target nucleic acid sequence or section thereof, the predetermined amount of control sequence added may be any suitable amount, preferably an amount within a factor of  $10^3$ -fold of the amount of target nucleic acid suspected to be contained within the sample. In a typical reaction it is likely that between 5 and  $10^5$  molecules of control sequence, eg. about 1000, will be used, although those of skill in the art will appreciate that this may vary, depending, for example, on the concentration of nucleic acid in the sample being analyzed. In order to achieve a more accurate result, a series of reactions each using a different amount of control sequence may be performed and the results compared.

- Amplification, preferably by means of PCR, is then carried out. Where PCR is employed, any number of amplification cycles that amplifies the target sequence to a sufficient degree may be used, for example up to 20, up to 40 or up to 80 cycles, with from 30 to 50 cycles being preferred and 35 to 45 cycles being particularly preferred in the first round of PCR. Similarly, any suitable conditions may be used, particularly as regards types and concentrations of reagents used in the PCR reactions and temperatures in the denaturation, annealing and extension stages of the PCR reaction.
- 10 In a particularly preferred embodiment of the invention, a nested PCR reaction is used to amplify the target and control sequences. In nested PCR, two or more pairs of primers are employed sequentially to amplify progressively shorter sections of the target and control sequences. Thus, in a two-stage nested PCR, two pairs of primers are used. The first pair hybridises to the target or control nucleic acid
- 15 towards the respective 5' ends of each strand. Amplification results in a nucleic acid product that is equivalent the section of the target or control sequence between the 5' ends of the two primers. A further pair of primers is then employed. These bind to the amplified product at sites that are 3' to the first primer binding sites and are thus within the first pair of primer binding sites.
- 20 Thus, the nucleic acid molecules of the primer pair used in each successive amplification routine are capable of hybridizing to the target sequence of SEQ. ID. No. 1 in such a position that the 5' ends of the primer regions are located at least one nucleotide 3' to the site at which the 5' end of the corresponding primer of the previous pair hybridized. Preferably, the 5' end of each successive
- 25 corresponding primer should hybridize to the target sequence at least 5, at least 10, at least 20 or at least 30 nucleotides 3' to the site at which the 5' end of the previous corresponding primer hybridized.

- A second or subsequent amplification stage results in a shorter nucleic acid section
- 30 than was obtained in the first amplification. The products of the second stage are then detected and quantified.

Nested PCR has the advantage that a greater total number of cycles of amplification can be performed without significant amplification of background contaminants (which are amplified to a small extent in many PCR reactions owing to non-specific binding of primers to sequences other than the one of interest).

5

This background amplification places a practical limit on the number of amplification cycles that can be achieved with any single primer pair. Nested PCR enables this limit to be overcome and lower levels of target sequence to be detected. This is particularly beneficial in clinical applications where HHV-7 may be present at very low levels in human tissues or bodily fluids. As an example of primers suitable for nested PCR according to the invention, primers HHV7-1 (SEQ. ID. No. 3) and HHV7-2 (SEQ. ID. No. 4) are suitable as the first (outer) pair of primers as they hybridise to sites close to the 5' ends of the two strands of the target sequence (SEQ. ID. No. 1). Primers HHV7-3 (SEQ. ID. No. 5) and HHV7-4 (SEQ. ID. No. 6) are suitable as the second (inner) pair of primers as they hybridise to sites further from the 5' ends of the two strands of the target sequence and more towards the centre of the target sequence. Second round amplification with these primers may occur for 5 to 40 cycles, with from 5 to 25 cycles being preferred and 12-20 cycles being particularly preferred.

20

Analysis of the products of the amplification reaction is then carried out. Quantitative analyses are preferred, though detection of the target nucleic acid sequence without quantification is also within the scope of the invention. Detection can, for example, be achieved using a single primer sequence of the invention as a probe. A single primer sequence will not act to amplify the target sequence but will, if it is sufficiently complementary, hybridise specifically to it. This specificity can be exploited, by any means known in the art (such as those of Sambrook *et al*; 1989, Molecular Cloning: A Laboratory Manual), to detect the presence of the target nucleic acid. For example, the probe may carry a label (e.g. a radioactive or fluorescent label) which can be detected by a suitable method.

30

Detection and quantification of the amplified target and control nucleic acids may

be performed by any suitable means known in the art. However, certain methods of detection and quantification are preferred. In particular, methods relying on the presence of a unique restriction site in either the target or control sequence are preferred, as described above. In such methods, one sequence, typically the control  
5 sequence, includes one or more restriction sites that are absent from the other sequence. Following amplification, one or more of these distinctive restriction sites is cleaved with the appropriate restriction enzyme. The cleavage products are then identified and quantified, thus allowing quantification of the target sequence. Alternatively, the presence of the altered base can be detected by a point mutation  
10 assay (Kaye *et al* (1992) J. Virol. Methods) to yield the distribution of bases at the specific position represented by target or control sequence amplicons.

Preferably, the PCR amplified target and control nucleic acids or fragments thereof, such as cleaved fragments of the control nucleic acid) are resolved by  
15 electrophoresis through other methods, eg. other types of size-fractionation may be used if appropriate.

Following resolution of the target and control nucleic acids, the relative amounts of these nucleic acids may be measured by any means known in the art.  
20 Preferably, the amplified nucleic acids will contain a radioactive label or fluorescent label which can be measured by, for example, autoradiography followed by scanning densitometry or laser detection of the amplified products. The radio label may be present in the primers, or incorporated in the nucleotides used in nucleic acid synthesis during amplification reaction.

25 Alternatively, the amplified target and control nucleic acids can be resolved by use of oligonucleotide probes which are specific for one or the other, but not both, amplified products. As the control nucleic acid typically differs slightly from the target sequence, it is possible to synthesise probes that are specific for the amplified  
30 control nucleic acid but do not, under conditions of high stringency, hybridise to the target nucleic acid. A second probe specific for the target sequence could be used to quantify that sequence. Alternatively, a first probe specific for the target



or control nucleic acid could be used to determine the quantity of one or other of these products and then a second probe specific to both products used on the same sample to ensure the total amount of nucleic acid produced. The difference between the measurement with the first probe and measurement with the probe  
5 common to both products will provide an indication of the amount of whichever of the selected and control products was not measured in the first measurement. In a further embodiment, where the difference between the control and target nucleic acids is small, a single oligonucleotide probe encompassing the region of difference and 100% homologous to a section of one or other of the two sequences  
10 could be used. At low stringency, the probe will hybridise to both the control and selected regions although at high stringency it will only hybridise to a region of 100% homology.

When amplification by PCR is used, the control and target nucleic acids will be  
15 substantially homologous. For example this may mean that besides the differences mentioned above which allow resolution of the amplified selected and control nucleic acids, there will be no other differences between the sequences. This will help to ensure equal efficiency of the reaction on both control and target sequences. However, minor alterations to the sequences may be possible without  
20 detriment to the present invention.

The nucleic acids which may be analyzed include DNA and RNA, with DNA being preferred. When RNA is being amplified by PCR, an initial reaction using a first primer and reverse transcriptase is required. The efficiency of reverse  
25 transcription can vary very significantly depending upon reaction conditions. Therefore, when the target nucleic acid is RNA, it is preferred that the control nucleic acid be RNA. Such control RNA can be generated using a control DNA sequence cloned into an RNA transcription vector which generates an RNA species using a suitable RNA polymerase in conjunction with a suitable promoter,  
30 eg a T3 or T7 promoter. The control RNA generated can be quantified using standard spectrophotometric assays. The advantage of such a system is that the efficiency of both the reverse transcription and PCR steps are controlled for.

The invention further provides isolated nucleic acid sequences related to the target and control sequences. The target and control sequences themselves are given in SEQ. ID. Nos. 1 and 2 respectively. The section of SEQ. ID. No. 1 amplified by the primers of SEQ. ID. Nos. 3 and 4 is given in SEQ. ID. No. 9, and the 143bp  
5 section of SEQ. ID. No. 1 amplified by primers of SEQ. ID. Nos. 5 and 6 is given in SEQ. ID. No. 10. The most preferred control sequence of the invention is given in SEQ. ID. No. 2. The section of SEQ. ID. No. 2 amplified by the primers of SEQ. ID. Nos. 5 and 6 is given in SEQ. ID. No. 11.

- 10 The invention also provides kits for quantification of HHV-7 in samples as described above. These kits comprise reagents suitable for performing methods as described above, and therefore for effecting detection, and preferably quantification, of HHV-7 in samples. A kit suitable for quantifying HHV-7 in a sample will comprise a control nucleic acid, for example the control nucleic acid of  
15 SEQ. ID. No. 2, as described above; and one or more pairs of primers as described above that are suitable for amplifying both the target and control nucleic acid sequences, or sections thereof. Preferred kits according to the invention comprise a control nucleic acid having a restriction site not present in the target nucleic acid, as described above; for example the control nucleic acid of SEQ. ID. No. 2, which  
20 contains a SmaI site.

Preferred kits of the invention may also comprise one or more preferred primer pairs such as the primer pair represented by SEQ. ID. Nos. 3 and 4 and/or the primer pair represented by SEQ. ID. Nos. 5 and 6. Particularly preferred kits of  
25 the invention will be suitable for performing nested PCR methods employing two or more pairs of primers. Thus, for example, a preferred kit according to the invention may comprise the primer pair represented by SEQ. ID. Nos. 3 and 4 together with the primer pair represented by SEQ. ID. Nos. 5 and 6. Typically, these will be provided in separate containers as they will be used in separate stages  
30 of a method according to the invention.

Kits according to the invention may also comprise any other suitable reagents, for

example a restriction enzyme, such as SmaI, suitable for cleaving a control sequence comprising a restriction site not found in the target nucleic acid.

5 Nucleic acids of the invention are preferably DNA although other nucleic acids such as RNA may be used.

The 193 base pair fragment of the invention encodes a polypeptide homologous with the KA3L gene of HHV-6. By extension, it is therefore the positional  
homologue of the Herpes Simplex Virus ICP27 gene. HSV ICP27 has been shown  
10 to be an important gene in the regulation of mRNA transcripts and it is therefore a potential antiviral target, since HSV ICP27 deletion mutants do not propagate in cell culture.

Therefore, the 193bp sequence, and the sequences of the invention that are related  
15 to it, may be useful in interrupting viral transcription. Accordingly, nucleic acid fragments having the sequences of SEQ. ID Nos 1 to 11, and the complementary strands thereof, may form the basis of antiviral agents.

In particular, they may be used to prepare "antisense" DNA constructs which,  
20 when transcribed as mRNA, hybridise to the "sense" mRNA transcribed from the corresponding native viral gene, typically a HHV-7 gene, thus forming a duplex that cannot be translated into a protein and is degraded by the host cell. Thus, the expression of the native viral protein is reduced, regulation of mRNA transcripts is disrupted, and viral replication in the host cell is impaired.

25

For *in vivo* activity, antisense oligonucleotides will require modifications in the molecule's backbone and possibly additions at either the 5' or 3', or both, ends of the molecule. This is to (1) achieve resistance to degradation by DNAases, (2) enhance the potency of the molecule and (3) to enhance uptake of the  
30 oligonucleotide by cells. This is an area of intense research and development. Currently the technology exists to produce sufficient quantities of modified oligonucleotides for therapeutic use (see for example Biotechnology, November

- 1993, page 1225). A number of different types of modification to oligonucleotides are known in the art. These include methylphosphonate and phosphorothioate backbones (where the phosphate groups in the nucleic acid backbone are replaced by methylene or sulphur) and addition of acridine or polylysine chains at the 3' and/or 5' end of the molecule. For the purpose of the present invention, it is to be understood that all or part of the oligonucleotides described herein may be modified by any method available in the art in order to enhance their in vivo activity or lifespan.
- 10 Further, it will be clear to those of skill in the art that specific oligonucleotides described herein are represented in standard notation, ie., by the letters A, C, G or T (or U) to indicate the base of the nucleotide, written in the 5' to 3' direction. Unless specified to the contrary, these sequences may be modified as described above.
- 15 The nucleic acid fragments (SEQ. ID Nos 1 to 11) of the invention may be used as the basis for antiviral agents against any virus that has a suitable sequence in its genome, preferably a human herpesvirus, more preferably HHV-7.
- 20 The invention therefore provides: nucleic acid fragments of the invention for use in methods of treatment of the human or animal body by therapy, particularly with a view to treating, or preventing or ameliorating pathologies caused by viruses, especially human herpesviruses, such as HHV-7; use of nucleic acid fragments of the invention in the manufacture of medicaments for the treatment, prevention or amelioration of such viral pathologies; methods of treating, preventing or ameliorating these viral diseases by administering nucleic acid sequences of the invention alone or in combination with a pharmaceutically acceptable carrier, diluent or excipient to human or animals in need thereof; and pharmaceutical compositions comprising nucleic acid sequences of the invention and a pharmaceutically acceptable carrier, diluent or excipient.
- 25  
30

The following examples illustrate the invention.

## EXAMPLES

### EXAMPLE 1

#### 5 Construction of Viral DNA Library

Viral DNA was extracted from infected cells by the method of Mukai *et al* (1994) (J. Med. Virol. 42, 224-227). Virus-infected cells were lysed and total nucleic acid digested with RNase a and DNase 1. Trichlorotrifluoroethane was added and mixed vigorously, after which the precipitate was pelleted by centrifugation at 400g  
10 for 5 min. Supernatant containing viral capsids was layered onto a discontinuous 5%/40% glycerol gradient and centrifuged at 100,000g for 90 minutes. The pellet was digested for 3 hours at 65°C using proteinase K in a 10 mM Tris.HCl, 1mM EDTA, 0.1% SDS buffer; yielding viral DNA which was purified by standard phenol:chloroform extraction and ethanol precipitation (Sambrook *et al* (1989)  
15 Molecular Cloning; a laboratory manual (2nd Ed): Cold Spring Harbour Laboratory Press, New York). Three microgrammes of viral DNA was digested with 20 units HindIII at 37°C for 18 hours in a volume of 50µl Ligation of fragments in pUC18 was achieved using a commercially-available kit (Ready-To-go, Pharmacia, England) according to the manufacturer's protocol. Competent bacterial  
20 host *E. coli* was transformed by standard protocols (Sambrook *et al*), and quantities of cloned HHV-7 DNA were used for sequence determination. Sequencing was performed by the dideoxy method of Sanger using M13 universal primers in standardized protocols (Sambrook *et al*). Comparison of sequences with those of other herpesviruses was carried out using the Genetics Computer Group software  
25 examination of GENBANK gene databases.

### EXAMPLE 2

#### (i) PCR Primers and Reaction Conditions

A suitable clone, 193bp in length and named 1D3 (SEQ. ID. No. 1) was selected  
30 and primer sequences delineating 183 bp (outer primers: SEQ. ID. Nos. 3 and 4) and 143 bp (inner primers: SEQ. ID. Nos. 5 and 6) of this sequence were synthesized (R&D Systems, Abingdon, England). PCR buffer composition was

16mM  $(\text{NH}_4)_2\text{SO}_4$ , 67mM Tris-HCl (pH 8.8 at 25°C, 0.010% Tween-20 (Bioline Ltd, London/England); 2 mM  $\text{MgSO}_4$ ; 100 ng each primer; 200  $\mu\text{M}$  dATP, dCTP, dGTP, dTTP; and 1 unit Taq polymerase ("BioTaq", Bioline, London, England). Total reaction volume was 50  $\mu\text{l}$  with 90  $\mu\text{l}$  mineral oil

- 5 overlay. Two rounds of PCR were performed, the first using the outer primers and the second using the inner primers. Thermal cycling conditions were the same for both rounds of PCR: 1 cycle at 95°C for 6 min; 39 cycles at 94°C, 50°C, 72°C for 30 seconds each followed by 72°C for 10 minutes.

10 (ii) Construction of Control Sequence

- Mutational primers HHV7-MUT5 (SEQ. ID. No. 7) and HHV7-MUT6 (SEQ. ID. No. 8) were designed spanning a suitable internal area (SEQ. ID. No. 2) of, but not entirely complementary to, the 1D3 sequence. Separate PCR reactions, employing respective outer primers and inner mutational primers (for example HHV7-1 and
- 15 HHV7-MUT6) were optimized and used to generate amplimers with overlapping sequences of the original 1D3 clone but with a SmaI site introduced (Figure 1, A). The two reaction products were mixed in equimolar proportions (Figure 1, B), denatured at 95°C for 10 minutes and allowed to reanneal at room temperature for 30 minutes (Figure 1, C). Klenow fragment of DNA polymerase was used to
- 20 synthesize the complementary strand from the overlap in the presence of 200 $\mu\text{M}$  dNTPs and enzyme buffer in a total volume of 100  $\mu\text{l}$  (Figure 1, D). After separation of the complete double-stranded product from excess primers, enzyme and dNTPs by using a commercially-available column (Qiaquick, Qiagen, England), the construct was further amplified by using the two complementary outer primers
- 25 (Figure 1, E). After similar purification the construct was ligated into the pUC18 plasmid vector (Ready-to-go, Pharmacia, England) followed by transformation of competent *E. coli* by standard protocols (Sambrook *et al*). Characterisation of suitable clones consisted of size analysis of excised fragments, linearization of plasmid by SmaI digestion, and sequencing of the inserts by the dideoxy method
- 30 of Sanger (Sambrook *et al*).

## (iii) Qualitative PCR on saliva samples (see Table 1)

PCR was performed, using the conditions described in (i) above, on saliva samples from 5 patients. The results are shown in Fig. 2. Following one round of amplification with the outer primers of SEQ. ID. Nos 3 and 4, a band was identified on an agarose gel. This corresponded in size to the 183bp fragment expected to result from the amplification.

Following a second round of amplification with the inner primers of SEQ. ID. Nos 5 and 6, a band corresponding in size to the expected 143bp fragment was strongly visualized on an agarose gel in all but one of the samples. This shows that the primers and methods of the invention readily detect HHV-7 DNA in practice.

All patients except the PCR negative individual were positive for HHV-7 antibodies using an immunofluorescence assay based on patient sera reactivity against HHV-7 injected SupT1 cells. Primers were used at standard protocol amounts (100 ng) and 30 $\mu$ l of whole saliva was used in a 50 $\mu$ l total PCR mixture.

**TABLE 1**

DETECTION OF HHV-6 AND HHV-7 IN SALIVA FROM HEALTHY VOLUNTEERS: COMPARISON WITH ANTIBODY STATUS

Volunteer No.	HHV-6		HHV-7	
	SALIVA PCR	SERO-STATUS	SALIVA PCR	SERO-STATUS
1	+	+	+	+
2	+	+	-	-
3	+	+	+	+
4	+	+	+	+
5	+	+	+	+

5	6	-	-	+	+
	7	+	+	+	+
	8	+	+	+	+
	9	+	+	+	+
	10	+	+	+	+
10	11	+	+	+	+
	12	+	+	+	+
	13	+	+	+	+
	14	+	+	+	+
	15	+	+	+	+
	16	+	+	+	+

Thirty microliters saliva tested by nested PCR for HHV-6 for HHV-6 and HHV-7.  
Sera screened at a dilution of 1:10 by indirect immunofluorescence assays.

15

### EXAMPLE 3

Standard curve and reproducibility of the quantitative assay.

The ability of the HHV-7 quantitative assay to give accurate data on samples with  
known genomic equivalents is illustrated in Figure 3. In this experiment, known  
copy numbers of plasmid containing the wild-type target ranging from 10 to  
1,000,000 copies were co-amplified with copy numbers of control sequence in the  
appropriate range (10-fold more or less than the target sequence). The calculated  
wild-type copy number was then plotted against the actual input copy number,  
with each data point representing a mean of three experiments. Linear regression  
analysis showed that the calculated and actual copy numbers were highly correlated  
( $R=0.994$ ;  $p<0.001$ ).



**EXAMPLE 4****Cross-sectional and longitudinal analysis of HHV-7 viral burden in blood**

- The prevalence of HHV-7 DNA in blood samples from HHV-7 seropositive individuals varies according to the quantity of input DNA used for analysis. Therefore, at inputs of 10 to 15 ng PBMC DNA the prevalence of HHV-7 DNA is between 10% and 12% of the seropositive population, whereas when 1  $\mu$ g of input DNA is used, 90% of seropositive individuals were found to contain HHV-7 DNA in their blood. These data are summarised in Figure 4. Using the PCR conditions inherent within this patent application, these results have implications for the detection of HHV-7 DNA in normal and in immunocompromised hosts to detect levels of viral DNA commensurate with reactivation/reinfection, rather than detection of latent viral DNA.
- Longitudinal analysis of HHV-7 viral load in the saliva and peripheral blood of healthy volunteers showed that the amount of virus remains relatively constant over time (see Figures 5 and 6).

**EXAMPLE 5****20 Detection of HHV-7 DNA in the blood of immunocompromised patients**

The qualitative and quantitative PCR methods described in this patent have been used to detect and measure HHV-7 in liver, bone marrow and renal transplant recipients, and HIV-infected patients.

25

- a) Qualitative presence of HHV-7 DNA in 40 ng of peripheral blood surveillance samples from immunocompromised patients.

- i) HHV-7 in transplant recipients

30

The results of the qualitative detection of HHV-7 in liver, renal and bone marrow transplant recipients on a sample and patient

basis are shown in Figures 7, 8 & 9, respectively. For comparative purposes, the detection rates for HHV-5 (cytomegalovirus) and HHV-6 are also shown in these Venn diagrams. HHV-7 was detected in 26/50 (52%) liver patients, 29/60 (48%) renal patients and 16/50 (34%) of the bone marrow transplant recipients. On a sample basis, 44/473 (9.3%) of samples from the liver patients, 53/729 (7.4%) of samples from the renal transplant patients, and 22/651 (3.4%) of samples from bone marrow transplant recipients were positive for HHV-7.

ii) HHV-7 in HIV-infected individuals.

The prevalence of HHV-7 in patients with HIV infections stratified according to CD4 cell numbers is shown in Table 2. A reduction in the overall prevalence of HHV-7 DNA in the peripheral blood was observed on progressing from a CD4 strata of  $>700 \mu\text{L}$  to  $<200 \mu\text{L}$ .

Table 2

DETECTION OF HHV-6 OR HHV-7 IN 473 SAMPLES COLLECTED FROM 160 HIV-POSITIVE PATIENTS

Number (%) samples PCR- positive for:	CD4 cells per cubic milimeter				TOTALS
	< 200	200-400	400-700	> 700	
HHV-6	8*/136(5.9%)	3/164(1.8%)	9/129(6.9%)	2/44(4.5%)	22/473(4.6%)
HHV-7	3*/136(2.2%)	1/164(0.61%)	4/129(3.1%)	3/44(6.8%)	11/473(2.3%)

\*One sample PCR-positive for both HHV-6 and HHV-7

b) HHV-7 load in liver transplant recipients

5 The maximum virus load detected during surveillance of liver transplant recipients is shown in Figure 9. Maximum viral loads for HHV-7 were similar to those for HHV-6, both of which were substantially lower than for CMV.

10 c) Clinicopathological Correlations of HHV-7 infection in liver transplant recipients

In contrast to other betaherpesviruses, our investigations have shown very few pathological consequences of HHV-7 infection in the liver transplant recipients to date.

15

EXAMPLE 6

Detection and quantification of HHV-7 DNA in children with febrile illness

20 Forty children aged between 6 months - 24 months with febrile illness have been investigated for the presence of HHV-7 DNA as a marker of primary infection relating to the clinical appearance of *exanthem subitum*-like disease. Six of these children had HHV-7 DNA in their blood, with a median viral load of 4,300,000 genomes/ml (range 57,000 - 290,000,000; Figure 10). Careful inspection

25 of the results of PCR on saliva from these individuals revealed that 3 were highly likely to be experiencing primary infection with HHV-7. These 3 children possessed the highest viral load of the group (median 32,000,000 genomes/ml), which was approximately 100-fold higher than the median viral load for HHV-6 in children undergoing primary HHV-6 infection (138,000 genomes/ml of blood).

30

EXAMPLE 7

Prevalence and quantity of HHV-7 in necropsy tissue from AIDS patients and

**control subjects**

DNA extracts (10 $\mu$ g) from organs obtained at necropsy were used as a substrate for HHV-7 DNA analysis by the qualitative and quantitative PCR methods described in this patent. Although HHV-7 DNA was detected in 59/125 (47%) of tissues from AIDS patients, it was at a significantly lower viral load than that presenting in control subjects (median HHV-7 load in controls 24 genomes/ $\mu$ g DNA; range 0 to 550,000), whereas in AIDS patients (median = 0 genomes / $\mu$ g DNA; range 0 to 295; Mann-Whitney test:  $p < 0.001$ ). These data are shown in Figure 11.

10

**Sequence Information****Target and Control Sequences**

SEQ. ID No. 1. 193 bp Target Sequence

15

AAGCTTTTTA CATTTGGCTT GCTTTTGGT TTGTAAATTC AATTGGACGG  
TTTGCTTAGA TTGCTGTGAA GCAAAGCTGC AAGACGGAGT TGTAGAACAT  
GCAACATTAA AGCTGTTGTA CTCTTCAGGC ATGTTAGGAT GCAGACCAAA  
CTCCATAAAT TCTTTGGGAA GATAGGTACA GAAATATAAG CTT

20

SEQ. ID No. 2. 183 bp Control Sequence

25

TTTTTACATT TGGCTTGCTT TTTGGTTTGT AAATTCAATT GGACGGTTTG  
CTTAGATTGC TGTGAAGCAA AGCTGCAACC CGGGGTTGTA GAACATGCAA  
CATTAAAGCT GTTGTACTCT TCAGGCATGT TAGGATGCAG ACCAAACTCC  
ATAAATTCTT TGGGAAGATA GGTACAGAAA TAT

30

**Outer (first round) primers**

HHV7-1 5' TTT TTA CAT TTG GCT TGC TTT TTG 3' (SEQ. ID No. 3)

HHV7-2 5' ATA TTT CTG TAC CTA TCT TCC CAA 3' (SEQ. ID No. 4).

**Inner (second round) primers**

HHV7-3 5' TGC TTT TTG GTT TGT AAA TTC 3' (SEQ. ID No. 5)

HHV7-4 5' GAA TTT ATG GAG TTT GGT CTG 3' (SEQ. ID No. 6)

### Mutational primers

HHV7-MUT5 5' CAA AGC TGC AAC CCG GGG TTG TAG AAC A 3'

5 (SEQ. ID No. 7)

HHV7-MUT6 5' TGT TCT ACA ACC CCG GGT TGC AGC TTT G 3'

(SEQ. ID No. 8)

### Amplified Sequences

10 SEQ. ID No. 9. The section of SEQ. ID No. 1 amplified by primers of SEQ. ID Nos. 3 and 4

TTTTTA CATTGGCTT GCTTTTGGT TTGTAAATTC AATTGGACGG  
TTTGCTTAGA TTGCTGTGAA GCAAAGCTGC AAGACGGAGT TGTAGAACAT  
15 GCAACATTAA AGCTGTTGTA CTCTTCAGGC ATGTTAGGAT GCAGACCAAA  
CTCCATAAAT TCTTTGGGAA GATAGGTACA GAAATAT

SEQ. ID No. 10. The section of SEQ. ID No. 1 amplified by primers of SEQ. ID Nos. 5 and 6

20

T GCTTTTGGT TTGTAAATTC AATTGGACGG  
TTTGCTTAGA TTGCTGTGAA GCAAAGCTGC AAGACGGAGT TGTAGAACAT  
GCAACATTAA AGCTGTTGTA CTCTTCAGGC ATGTTAGGAT GCAGACCAAA  
CTCCATAAAT TC

25

SEQ. ID No. 11. The section of SEQ. ID No. 2 amplified by primers of SEQ. ID Nos. 5 and 6

CAA AGCTGCAACC CGGGGTTGTA GAACA

30

## CLAIMS

1. An isolated nucleic acid molecule comprising:
  - (i) a primer portion consisting of a contiguous sequence of from 10 to 50 nucleotides capable of hybridising to (a) the target nucleic acid molecule represented by SEQ. ID No. 1, or (b) to the complementary stand thereof; and optionally
  - (ii) a further portion comprising from 1 to 25 nucleotides joined to and immediately 5' to the 5' end of the primer portion.
2. A pair of nucleic acid molecules according to claim 1 wherein one nucleic acid molecule has a primer region of type (a) as defined in claim 1 and the other nucleic acid molecule has a primer region of type (b) as defined in claim 1; and wherein the two primers in combination are capable of amplifying the target nucleic acid molecule represented by SEQ. ID No. 1, or a section thereof, in a polymerase chain reaction.
3. A pair of nucleic acid molecules according to claim 2 that, respectively, hybridise to the target nucleic acid sequence of SEQ. ID No. 1 and its complementary stand in such positions that the 5' end of the each primer region hybridises at a location that is from 0 to 50 nucleotides 3' to the 5' end of the sequence of SEQ. ID No. 1 or the complementary strand thereto.
4. A nucleic acid molecule according to claim 1 selected from the molecules represented by SEQ. ID Nos. 3, 4, 5 and 6.
5. A pair of nucleic acid molecules according to claim 2 wherein the nucleic acid molecule of type (a) is the nucleic acid molecule of SEQ. ID No. 3 and the nucleic acid molecule of type (b) is the nucleic acid molecule of SEQ. ID No.

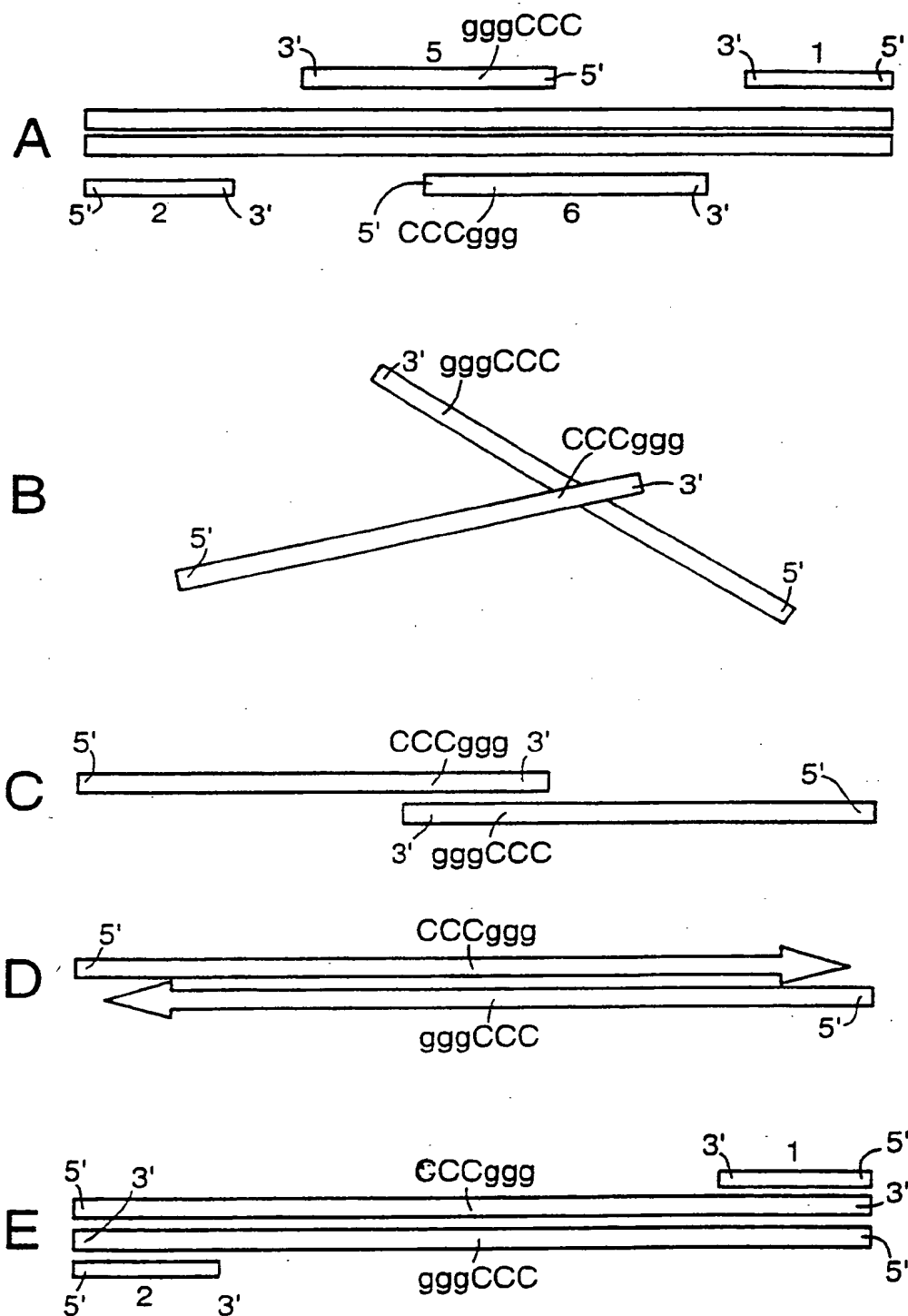
- 4.
6. A pair of nucleic acid molecules according to claim 2 wherein the nucleic acid molecule of type (a) is the nucleic acid molecule of SEQ. ID No. 5 and the nucleic acid molecule of type (b) is the nucleic acid molecule of SEQ. ID No. 6.
7. A method of determining the amount of a target nucleic acid having the sequence shown in SEQ. ID No. 1 in a sample which method comprises:
  - (i) mixing the sample with a predetermined amount of control nucleic acid;
  - (ii) bringing the mixture formed in (i) into contact with a pair of nucleic acid molecules according to any one of claims 2, 3, 5 or 6;
  - (iii) performing a nucleic acid amplification reaction, said reaction requiring the presence of the primers defined in (ii) to amplify the target nucleic acid sequence or a section thereof and the control nucleic acid or a section thereof;
  - (iv) determining the relative quantities of the amplified control and target nucleic acids; and
  - (v) calculating from the determination of (iv) the amount of target nucleic acid in the sample.
8. A method according to claim 7 wherein amplification is performed by means of a polymerase chain reaction (PCR).
9. A method according to claim 9 wherein the control sequence comprises at least one restriction site not present in the target sequence and wherein the determination performed in step (iv) comprises the step of cleaving one or more such restriction sites.
10. A method according to claim 9 wherein the restriction site is a SmaI site.

11. A method according to claim 10 wherein the control sequence comprises the sequence shown in SEQ ID No. 2 or any section thereof containing a restriction site not present in the sequence of SEQ ID No. 1.
12. A method according to any one of claims 7 to 11 wherein contacting step (ii) and amplification step (iii) are performed at least one further time, each time using a further pair of nucleic acid molecules according to any one of claims 2, 3, 5 or 6; the nucleic acid molecules of the pair used in each successive contacting and amplification routine being capable of hybridising to the target sequence of SEQ. ID No. 1 in such a position that the 5' ends of the primer regions are located at least one nucleotide 3' to the site at which the 5' end of the previous pair hybridises; such that the PCR performed is a nested PCR.
13. A method according to claim 12 wherein steps (ii) and (iii) are performed twice, with the nucleic acid pair of claim 5 being employed in the first round and the nucleic acid pair of claim 6 being employed in the second round.
14. An isolated nucleic acid molecule having a sequence selected from the sequences shown in SEQ ID Nos. 1, 2, 9, 10 and 11; or the complementary strands thereof.
15. A kit for quantification of human herpesvirus 7 (HHV-7) in a sample by means of a method according to any one of claims 7 to 13, which kit comprises:
  - (i) a control nucleic acid as defined in any one of claims 7 to 11;
  - (ii) a pair of primers according to any one of claims 2, 3, 5 and 6 that are suitable for amplifying both the target and control nucleic acid sequences, or sections thereof.
16. A kit according to claim 15 which further comprises (iii) one or more pairs of nucleic acids suitable for use as further pairs of nucleic acids in a method according to claim 12.



17. A kit according to claim 16 comprising
  - (i) a control sequence of SEQ. ID No. 2,
  - (ii) a pair of nucleic acids according to claim 5; and
  - (iii) a pair of nucleic acids according to claim 6; which kit is suitable for performing a method according to claim 13.
18. A nucleic acid molecule according to claim 1 or 14 or having a sequence as shown in any one of SEQ. ID. Nos 3 to 8 for use in a method of treating, preventing or ameliorating a pathology caused by a human herpesvirus.
19. A nucleic acid molecule according to claim 18 where the herpesvirus is HHV-7.
20. A pharmaceutical composition comprising a nucleic acid molecule according to claim 1 or 14, or having the sequence shown in any one of SEQ. ID Nos. 3 to 8, or a sequence complementary thereto; and a pharmaceutically acceptable carrier.

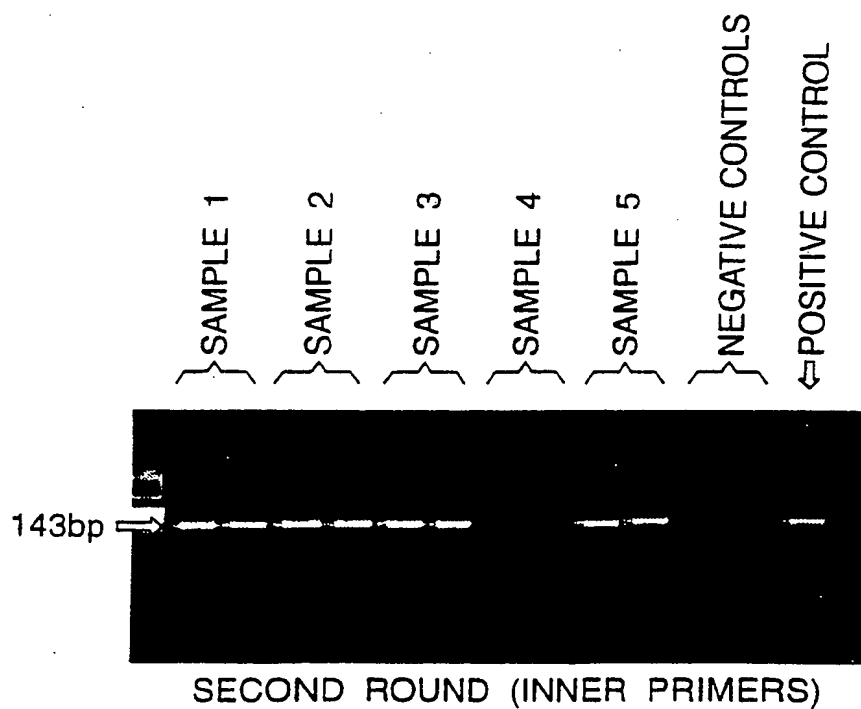
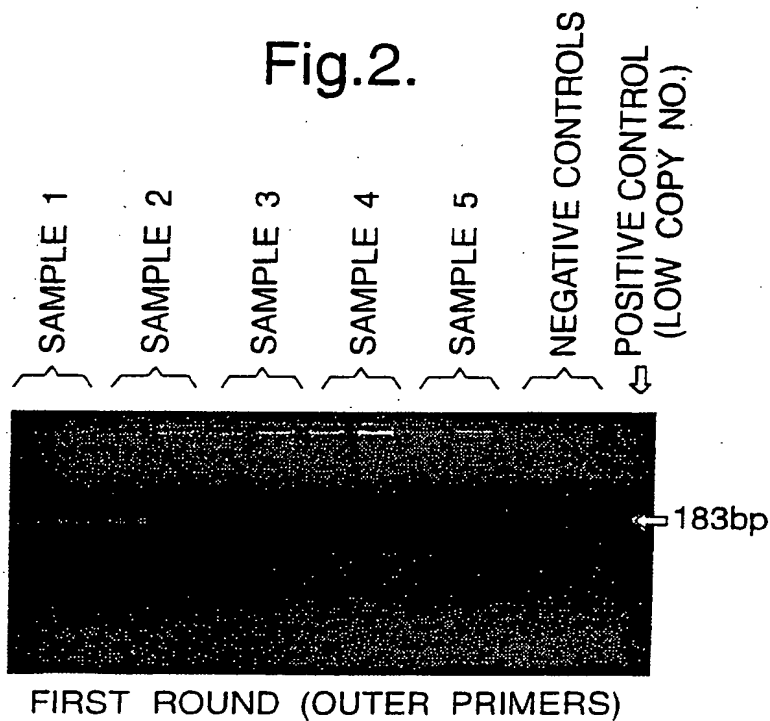
Fig.1.



SUBSTITUTE SHEET (RULE 26)

2/6

Fig.2.



SUBSTITUTE SHEET (RULE 26)

3/6

Fig.3.

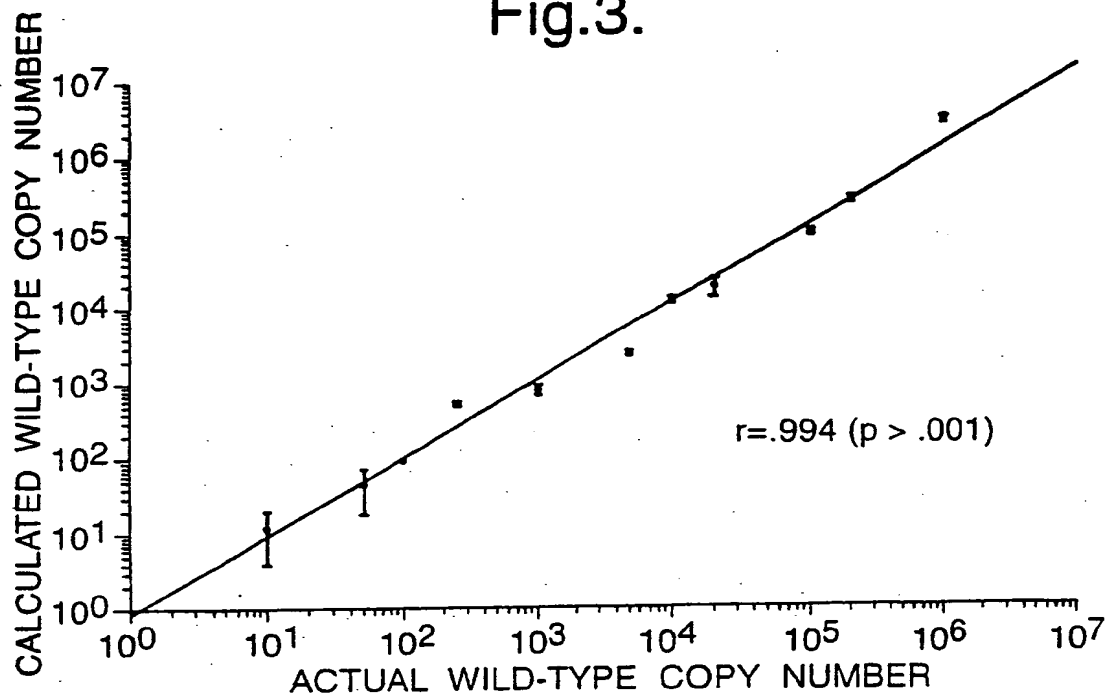
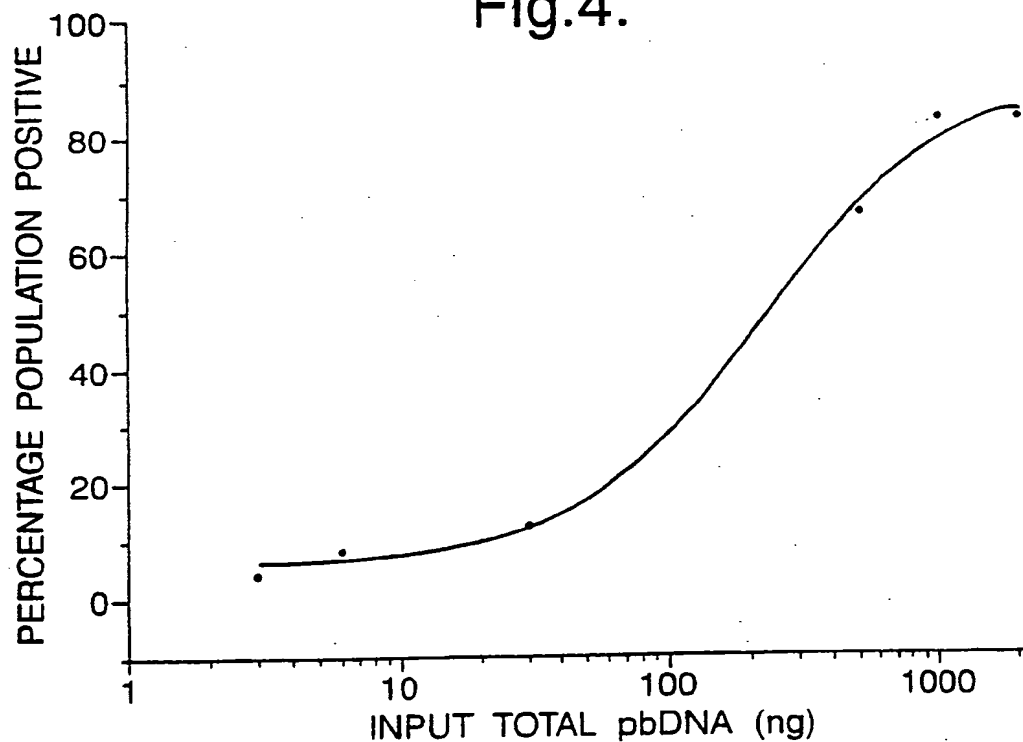


Fig.4.



SUBSTITUTE SHEET (RULE 26)

4/6

Fig.5.

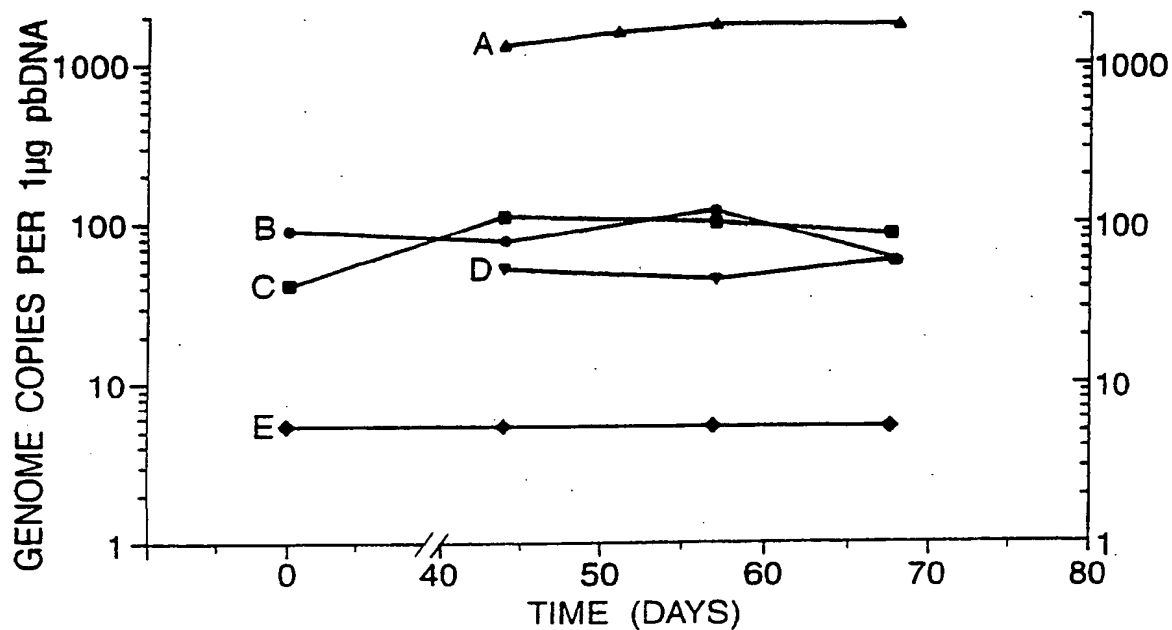
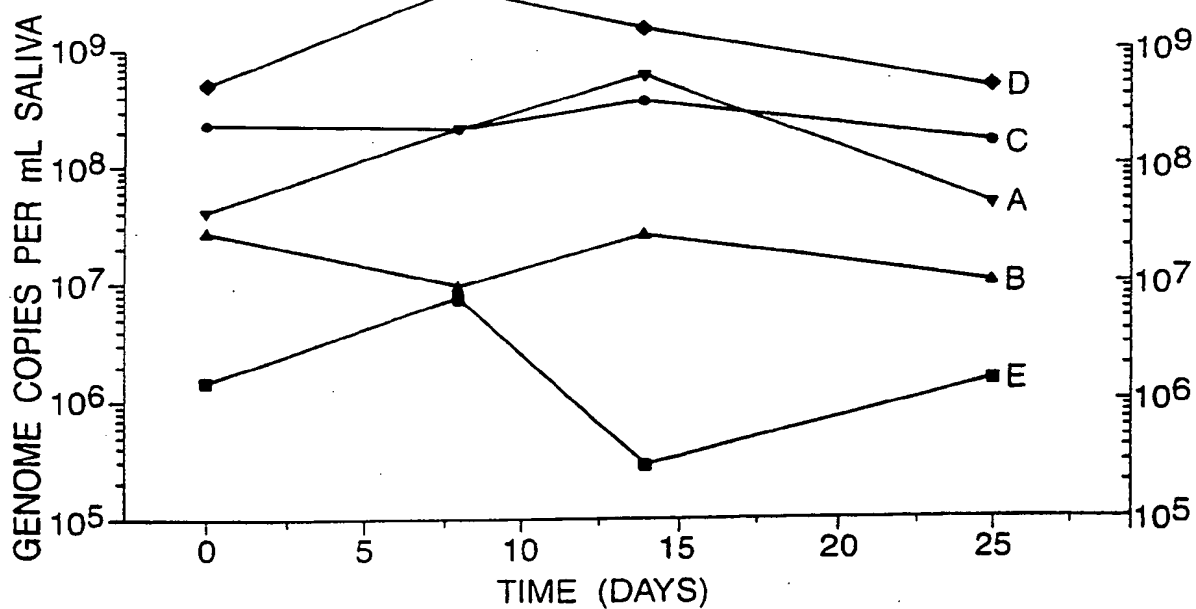


Fig.6.



SUBSTITUTE SHEET (RULE 26)

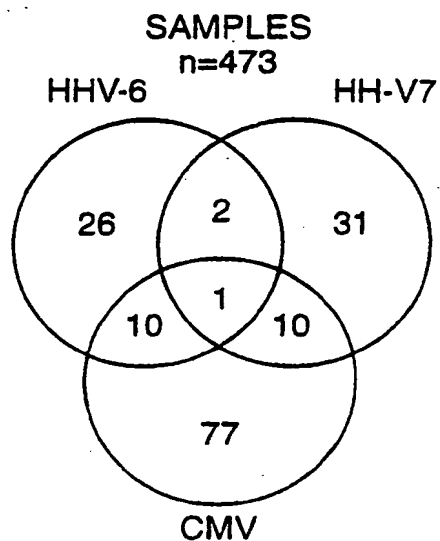
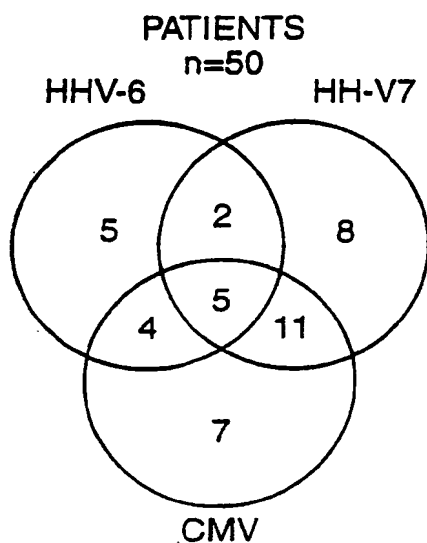
5/6  
Fig.7.

Fig.8.

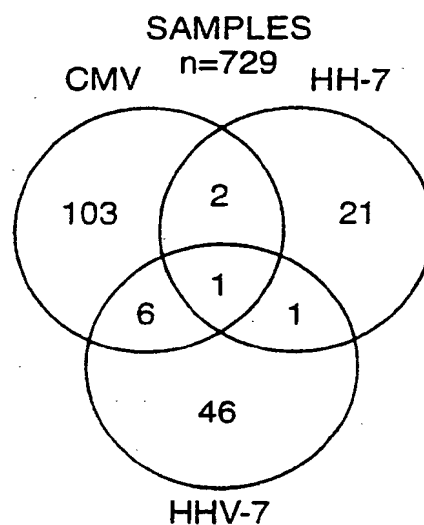
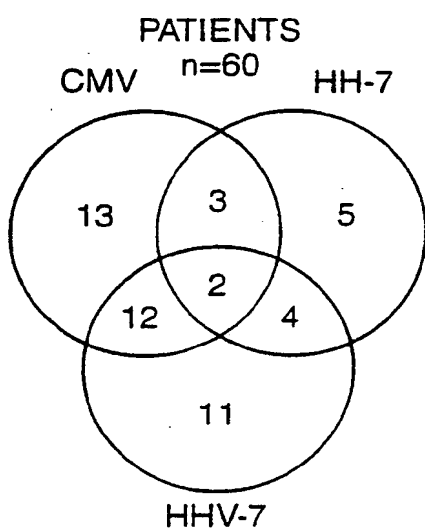
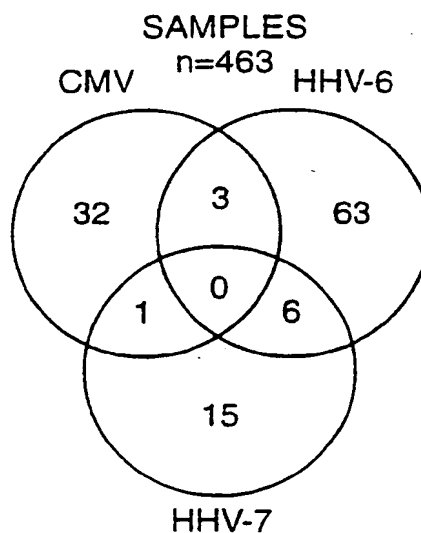
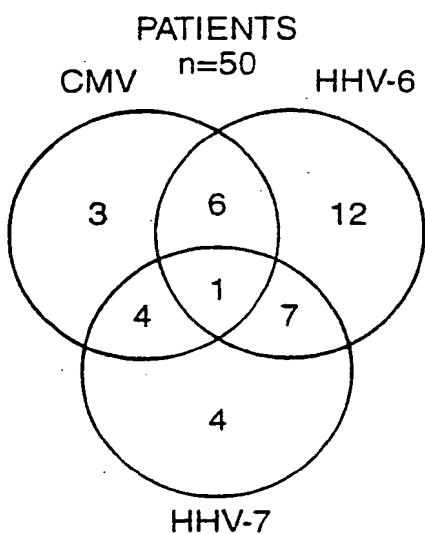


Fig.9.



SUBSTITUTE SHEET (RULE 26)

6/6

Fig.10.

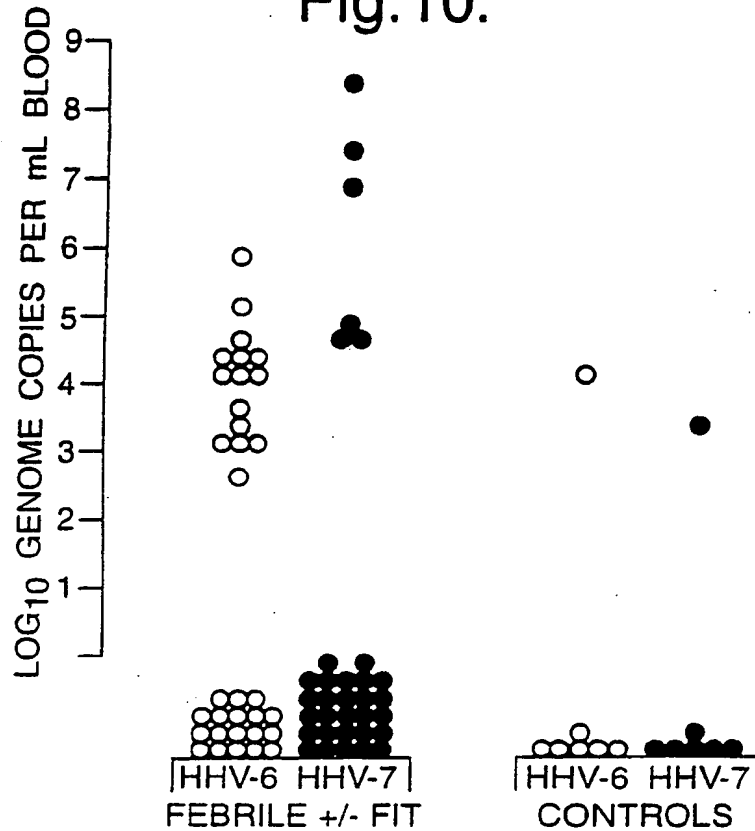
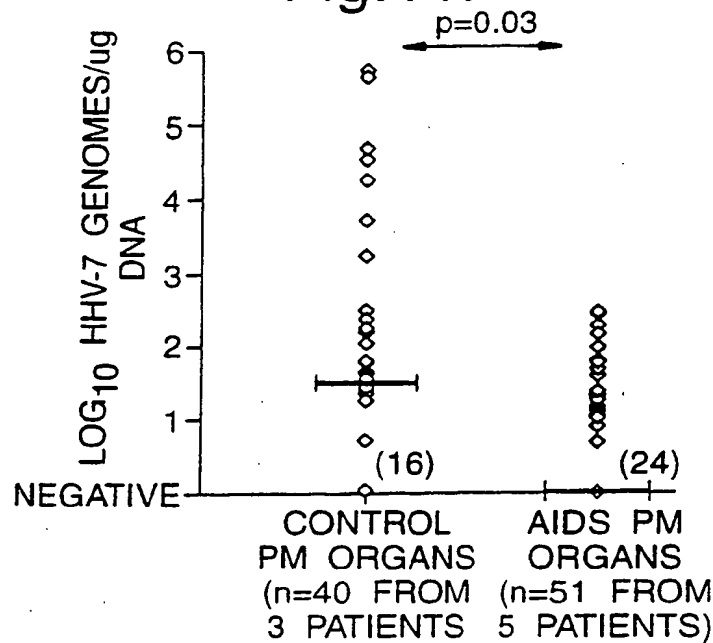


Fig.11.



SUBSTITUTE SHEET (RULE 26)

**THIS PAGE BLANK (USPTO)**

**THIS PAGE**



**PCT**WORLD INTELLECTUAL PROPERTY ORGANIZATION  
International Bureau

## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification <sup>6</sup> :</b> <b>C12Q 1/70, 1/68, A61K 31/70</b>	<b>A3</b>	<b>(11) International Publication Number:</b> <b>WO 97/03345</b> <b>(43) International Publication Date:</b> 30 January 1997 (30.01.97)
<b>(21) International Application Number:</b> PCT/GB96/01660 <b>(22) International Filing Date:</b> 12 July 1996 (12.07.96)  <b>(30) Priority Data:</b> 9514325.1 13 July 1995 (13.07.95) GB  <b>(71) Applicant (for all designated States except US):</b> ROYAL FREE HOSPITAL SCHOOL OF MEDICINE [GB/GB]; Rowland Hill Street, London NW3 2PF (GB).  <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only):</b> EMERY, Vincent, Clive [GB/GB]; Department of Virology, Division of Pathology & Communicable Diseases, Rowland Hill Street, London NW3 2PF (GB). GRIFFITHS, Paul [GB/GB]; Department of Virology, Division of Pathology & Communicable Diseases, Rowland Hill Street, London NW3 2PF (GB).  <b>(74) Agents:</b> BRASNETT, Adrian, Hugh et al.; J.A. Kemp & Co., 14 South Square, Gray's Inn, London WC1R 5LX (GB).		<b>(81) Designated States:</b> JP, US, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).  <b>Published</b> <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>  <b>(88) Date of publication of the international search report:</b> 13 March 1997 (13.03.97)
<b>(54) Title:</b> DETECTION AND QUANTIFICATION OF HUMAN HERPESVIRUS 7 BY ENZYMATIC AMPLIFICATION OF DNA VIRUS DNA <b>(57) Abstract</b>  An isolated nucleic acid molecule comprising: (i) a primer portion consisting of a contiguous sequence of from 10 to 50 nucleotides capable of hybridising to (a) the target nucleic acid molecule represented by SEQ. ID No. 1, or (b) to the complementary stand thereof; and optionally (ii) a further portion comprising from 1 to 25 nucleotides joined to and immediately 5' to the 5' end of the primer portion.		

**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AM	Armenia	GB	United Kingdom	MW	Malawi
AT	Austria	GE	Georgia	MX	Mexico
AU	Australia	GN	Guinea	NE	Niger
BB	Barbados	GR	Greece	NL	Netherlands
BE	Belgium	HU	Hungary	NO	Norway
BF	Burkina Faso	IE	Ireland	NZ	New Zealand
BG	Bulgaria	IT	Italy	PL	Poland
BJ	Benin	JP	Japan	PT	Portugal
BR	Brazil	KE	Kenya	RO	Romania
BY	Belarus	KG	Kyrgyzstan	RU	Russian Federation
CA	Canada	KP	Democratic People's Republic of Korea	SD	Sudan
CF	Central African Republic	KR	Republic of Korea	SE	Sweden
CG	Congo	KZ	Kazakhstan	SG	Singapore
CH	Switzerland	LI	Liechtenstein	SI	Slovenia
CI	Côte d'Ivoire	LK	Sri Lanka	SK	Slovakia
CM	Cameroon	LR	Liberia	SN	Senegal
CN	China	LT	Lithuania	SZ	Swaziland
CS	Czechoslovakia	LU	Luxembourg	TD	Chad
CZ	Czech Republic	LV	Latvia	TG	Togo
DE	Germany	MC	Monaco	TJ	Tajikistan
DK	Denmark	MD	Republic of Moldova	TT	Trinidad and Tobago
EE	Estonia	MG	Madagascar	UA	Ukraine
ES	Spain	ML	Mali	UG	Uganda
FI	Finland	MN	Mongolia	US	United States of America
FR	France	MR	Mauritania	UZ	Uzbekistan
GA	Gabon			VN	Viet Nam

# INTERNATIONAL SEARCH REPORT

International Application No.

PCT/GB 96/01660

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12Q1/70 C12Q1/68 A61K31/70

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	JOURNAL OF MEDICAL VIROLOGY, vol. 45, March 1995, pages 282-83, XP000614412 PORTOLANI M ET AL: "Isolation of human Herpesvirus 7 from an infant with Febrile Syndrome" see the whole document ---	7-17
Y	ARCHIVES OF VIROLOGY, vol. 136, 1994, pages 183-90, XP000614406 YALCIN S ET AL: "Human herpesvirus 6 and human herpesvirus 7 infections in renal transplant recipients and healthy adults in Turkey" see the whole document ---	7-17
	-/--	

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

### \* Special categories of cited documents:

- \*A\* document defining the general state of the art which is not considered to be of particular relevance
- \*E\* earlier document but published on or after the international filing date
- \*L\* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- \*O\* document referring to an oral disclosure, use, exhibition or other means
- \*P\* document published prior to the international filing date but later than the priority date claimed

\*T\* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

\*X\* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

\*Y\* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

\*&\* document member of the same patent family

Date of the actual completion of the international search

24 January 1997

Date of mailing of the international search report

1 2. 02. 97

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2  
NL - 2280 HV Rijswijk  
Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl,  
Fax: (+ 31-70) 340-3016

Authorized officer

Osborne, H

# INTERNATIONAL SEARCH REPORT

Internal Application No  
PCT/GB 96/01660

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	<p>PEDIATRICS, vol. 95, no. 2, February 1995, pages 187-90, XP000614347 ASANO Y ET AL: "Clinical features and viral excretion in an infant with primary human herpesvirus 7 infection" see the whole document ---</p>	7-17
Y	<p>WO,A,94 10344 (THE GOVERNMENT OF THE UNITED STATES OF AMERICA) 11 May 1994 see the whole document ---</p>	7-17
Y	<p>HEPATOLOGY, vol. 19, no. 5, May 1994, pages 871-75, XP000614397 NAITO M ET AL: "Serum hepatitis c virus RNA quantity and histological features of hepatitis c virus carriers with persistently normal ALT levels" see page 862, paragraph 3 - page 863, paragraph 2 ---</p>	7-17
Y	<p>GENETIC ANALYSIS TECHNIQUES AND APPLICATIONS, vol. 11, no. 1, 1994, page 1-6 XP000446393 CLEMENTI M ET AL: "competitive polymerase chain reaction and analysis of viral activity at the molecular level" see page 3, paragraph 2 ---</p>	7-17
A	<p>WO,A,92 01816 (THE UNITED STATES OF AMERICA) 6 February 1992 see claims 13,15 ---</p>	7-17,20
X	<p>WO,A,94 00140 (LUDWIG ISTITUTE FOR CANCER RESEARCH) 6 January 1994 see SEQ ID NO 145 -----</p>	1

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/GB 96/ 01660

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:  
Please see Further Information sheet enclosed.
2. ☐ Claims Nos.:  
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

# INTERNATIONAL SEARCH REPORT

International Application No. PCT/GB 96/ 01660

FURTHER INFORMATION CONTINUED FROM PCT/ISA/210

Remark: Although claims 18, 19 as far as they concern an in vivo method, are directed to a method of treatment of the human/animal body the search has been carried out and based on the alleged effects of the compound/composition.

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/GB 96/01660

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-9410344	11-05-94	AU-A- 5447194	24-05-94
WO-A-9201816	06-02-92	US-A- 5230997	27-07-93
		AU-A- 8406591	18-02-92
WO-A-9400140	06-01-94	US-A- 5530109	25-06-96
		AU-A- 4769493	24-01-94
		CA-A- 2139136	06-01-94
		EP-A- 0653940	24-05-95
		JP-T- 8509357	08-10-96
		PT-A- 101297	30-06-94
		ZA-A- 9304711	14-06-94

**THIS PAGE BLANK (USPTO)**



**This Page is Inserted by IFW Indexing and Scanning  
Operations and is not part of the Official Record**

**BEST AVAILABLE IMAGES**

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☐ **BLACK BORDERS**
- ☐ **IMAGE CUT OFF AT TOP, BOTTOM OR SIDES**
- ☐ **FADED TEXT OR DRAWING**
- ☐ **BLURRED OR ILLEGIBLE TEXT OR DRAWING**
- ☐ **SKEWED/SLANTED IMAGES**
- ☒ **COLOR OR BLACK AND WHITE PHOTOGRAPHS**
- ☐ **GRAY SCALE DOCUMENTS**
- ☐ **LINES OR MARKS ON ORIGINAL DOCUMENT**
- ☐ **REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY**
- ☐ **OTHER:** \_\_\_\_\_

**IMAGES ARE BEST AVAILABLE COPY.**

**As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.**

**THIS PAGE BLANK (USPTO)**